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# THE COEVOLUTION OF BACTERIAL ENDOSYMBIONTS AND PHLOEM-FEEDING INSECTS<sup>1</sup>

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## ABSTRACT

Insects that feed on plant sap depend on symbiotic bacteria for nutrients that are not present in the diet. These bacteria live within host cells and are transmitted from mother to offspring. The symbionts of aphids, in the genus *Buchnera*, are the best characterized of insect endosymbionts. They result from an ancient infection of a common ancestor of modern aphids, and they possess genes underlying pathways for production of essential amino acids that are rare in the phloem sap diet of aphids. Thus these bacteria appear to be highly coadapted with hosts and to have evolved as mutualists for millions of years. Nonetheless, *Buchnera* and other endosymbionts possess some genetic traits that appear to be deleterious to both hosts and, ultimately, the bacterial symbionts themselves. The most likely basis for these traits is the fixation of slightly deleterious mutations in the context of genetic drift in these bacterial populations, which possess small genetic population sizes relative to many free-living bacteria. Investigations during the next few years will reveal the extent of convergence among independently derived symbionts of different insect families and the extent to which symbionts are adapted to species-specific aspects of their host's ecology and nutrition.

*Key words:* aphid, *Buchnera*, coevolution, endosymbiont, mutualism, phloem sap, phylogenetics.

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## INTRODUCTION: PHLOEM-FEEDING AND ANIMAL NUTRITION

Among the major groups of organisms, animals stand out for their limited biosynthetic capabilities. Many vitamins and amino acids cannot be synthesized by animals, which must ingest each of these compounds in the diet. As a result, dietary specialization imposes the risk of nutritional deficiencies.

Several groups of insects feed only on the phloem sap of plants. Because tapping into a single phloem sieve element allows access to nutrients from throughout the plant without moving, this feeding mode is potentially rewarding. Furthermore, phloem sap of plants is rich in sugars and contains free amino acids. However, the nutrient profile of phloem is not favorable to animal growth. Although nitrogen is present in the form of free amino acids, there is a deficit of essential amino acids, the set required in animal diets (Fig. 1; Sandström & Moran, 1999). Whereas plant and animal proteins show similar amino acid abundance profiles, phloem sap contains primarily a few non-essential amino acids, which are transaminated as needed for protein synthesis at the site of growth. Thus, the reproduction and development of phloem feeders may be limited by certain essential amino

acids, causing excess nonessential amino acids to be excreted as waste (Sandström & Moran, 1999). In addition, phloem may be deficient in some vitamins.

Phloem-feeding insects have adopted a common solution to nutritional deficiencies: association with bacterial endosymbionts that have the capacity for biosynthesis of required amino acids (P. Baumann et al., 1999). These bacteria are confined to specialized host cells called bacteriocytes (or mycetocytes) that together form an organ called a bacteriome (Moran & Telang, 1998). The bacteria are transmitted maternally with progeny inoculated before oviposition or birth. Bacteriocyte associates appear to be present in all strict phloem-feeders, including most members of the clade corresponding to suborder Sternorrhyncha and many members of the suborder Auchenorrhyncha. These two suborders within the Hemiptera are often lumped as Homoptera, although not a monophyletic group (von Dohlen & Moran, 1995). The Sternorrhyncha includes aphids (Aphidoidea), scale insects (Coccoidea), whiteflies (Aleyrodoidea), and psyllids (Psylloidea) (Fig. 2). Current understanding of the genetics and evolution of the aphid-symbiont association is more extensive than for any other animal endosymbiosis. More limited knowledge for psyllids, whiteflies, and some scale insects suggests

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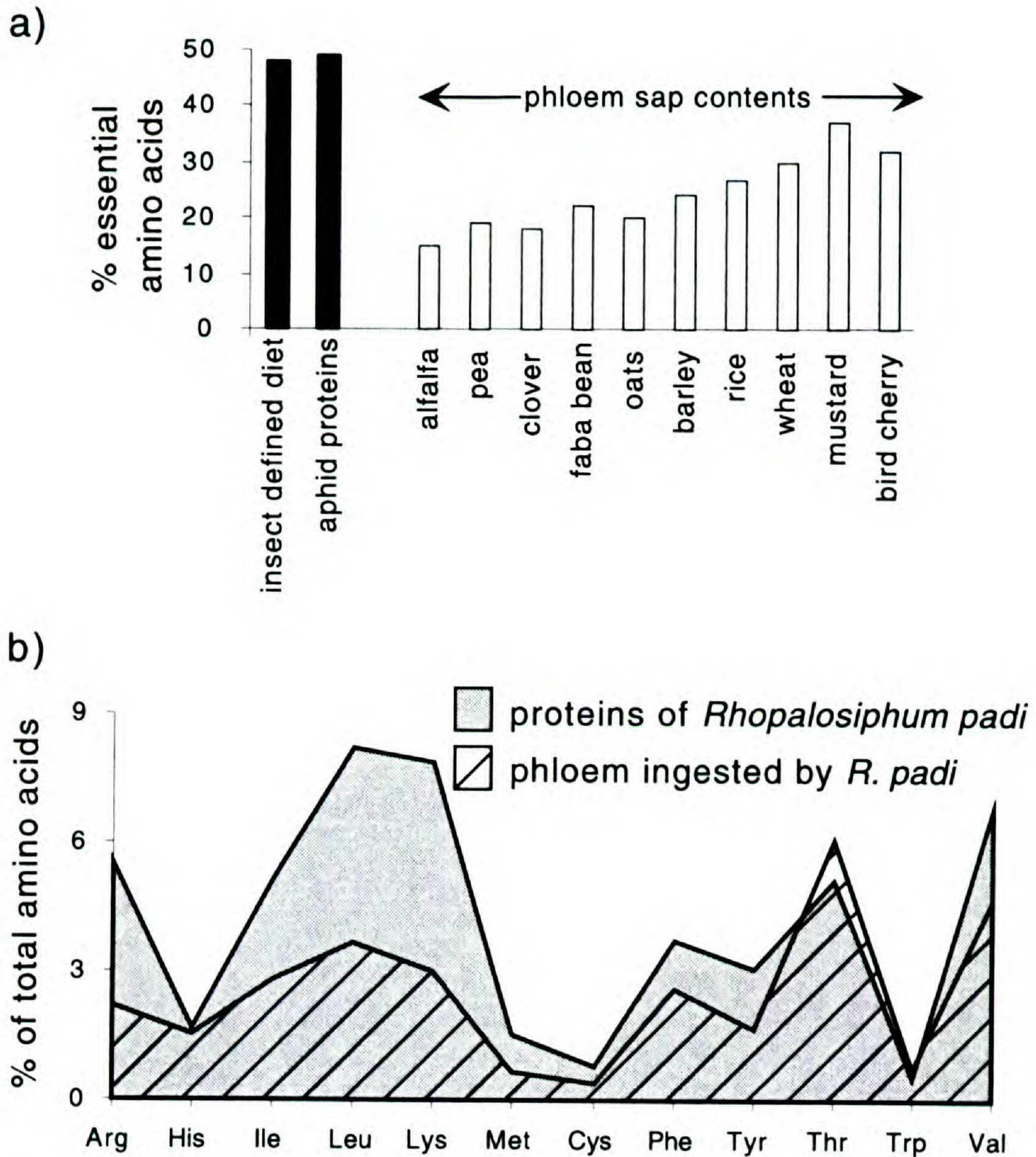


Figure 1. Quality of phloem sap as a diet for aphids. —a. Proportion of essential amino acids in aphid proteins, insect-defined diets, and phloem sap of various plants as sampled from aphid stylets. In phloem sap, a higher proportion of amino acids are nonessential. —b. Proportions of individual essential amino acids of total amino acids in the diet and body proteins of the aphid *Rhopalosiphum padi*. Most essential amino acids are deficient relative to requirements for protein synthesis. (Although amino acid profiles of proteins are roughly representative of dietary requirements, some, including tryptophan, are required in higher concentrations due to use in other endproducts or due to differences in efficiency of absorption). Data are summarized from Sandström and Moran (1999) for various studies based on phloem sap ingested by different aphid species on several angiosperm species (alfalfa = *Medicago sativa* L.; pea = *Pisum sativum* L.; clover = *Trifolium pratense* L.; faba bean = *Vicia faba* L.; oats = *Avena sativa* L.; barley = *Hordeum vulgare* L.; rice = *Oryza sativa* L.; wheat = *Triticum aestivum* L.; mustard = *Brassica juncea* (L.) Czern.; bird cherry = *Prunus padus* L.).

that similar patterns of evolution will extend at least to these groups, and some parallels have been observed in other invertebrate endosymbioses, including cockroaches (Bandi et al., 1995), carpenter ants (Schröder et al., 1996), tsetse flies (Chen et al., 1999), and bivalves (Peek et al., 1998).

In this paper, I present an overview of the current knowledge of the coevolution of endosymbionts with hosts in the Sternorrhyncha, especially aphids.

#### BIOLOGICAL BACKGROUND

Aphid primary endosymbionts are classified as *Buchnera aphidicola* (Munson et al., 1991a) and

belong to the gamma-Proteobacteria, near *Escherichia coli* and other enterics (Munson et al., 1991b). *Buchnera* lives within maternal cells called bacteriocytes, and infection occurs within the mother's body, during the egg or embryo stage (Buchner, 1965). Increase in bacterial numbers during development closely tracks the increase in aphid body size (Baumann & Baumann, 1994). Nutritional studies suggest that *Buchnera* provisions the host with certain amino acids (Douglas, 1998), and genetic studies have confirmed that the enzymatic pathways for amino acid biosynthesis are retained and modified to allow increased pro-

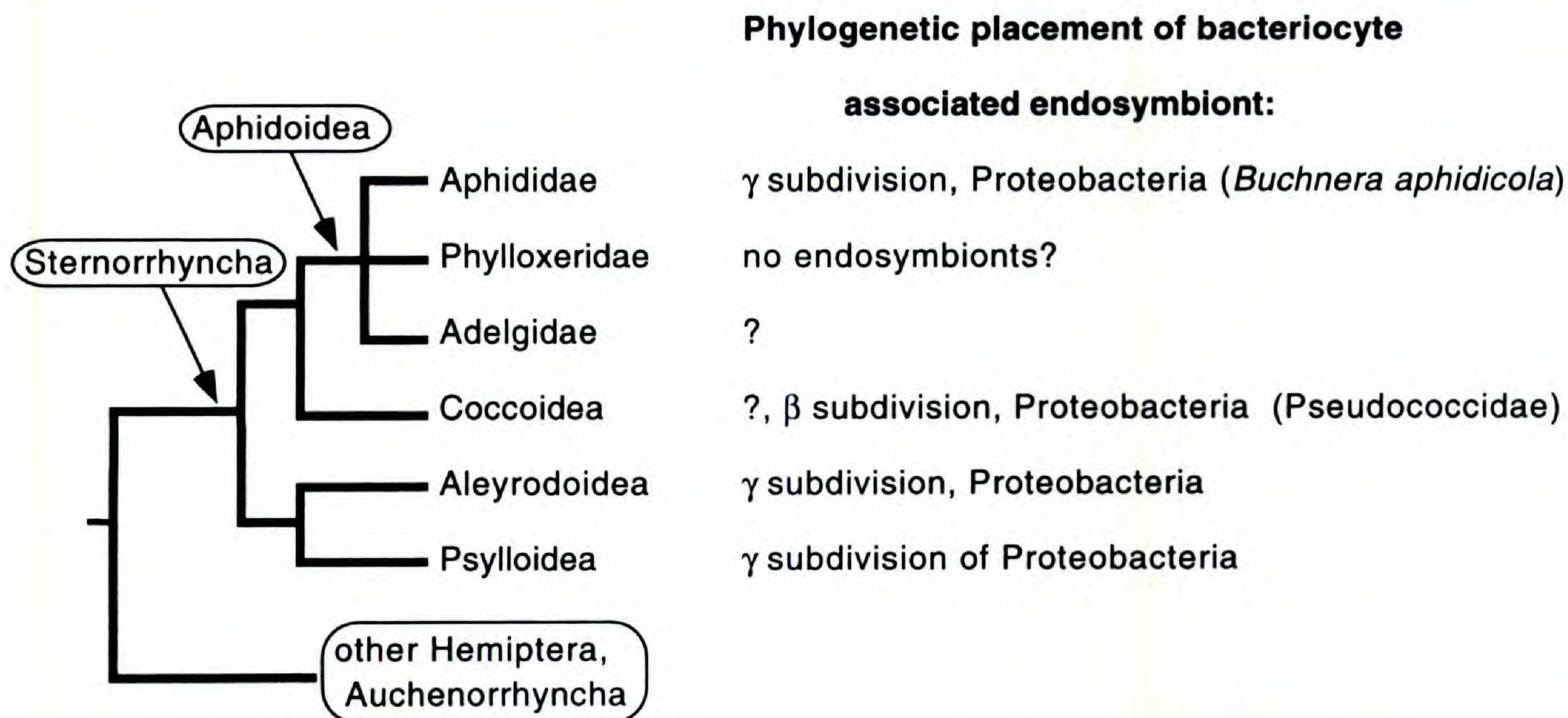


Figure 2. Phylogenetic relationships within the Sternorrhyncha (von Dohlen & Moran, 1995) and affiliations, where known, of bacteriocyte-associated or “primary” symbionts of major groups (Buchner, 1965; Munson et al., 1991b; Clark et al., 1993; Spaulding & von Dohlen, 1998; Thao et al., 2000a).

duction of end products (Lai et al., 1994; Bracho et al., 1995).

Other Sternorrhyncha also possess endosymbionts that live within bacteriocytes and that are transmitted maternally. However, the phylogenetic position of the symbionts, mechanisms of vertical transmission, and the position and characteristics of bacteriocytes differ among major groups of insect hosts.

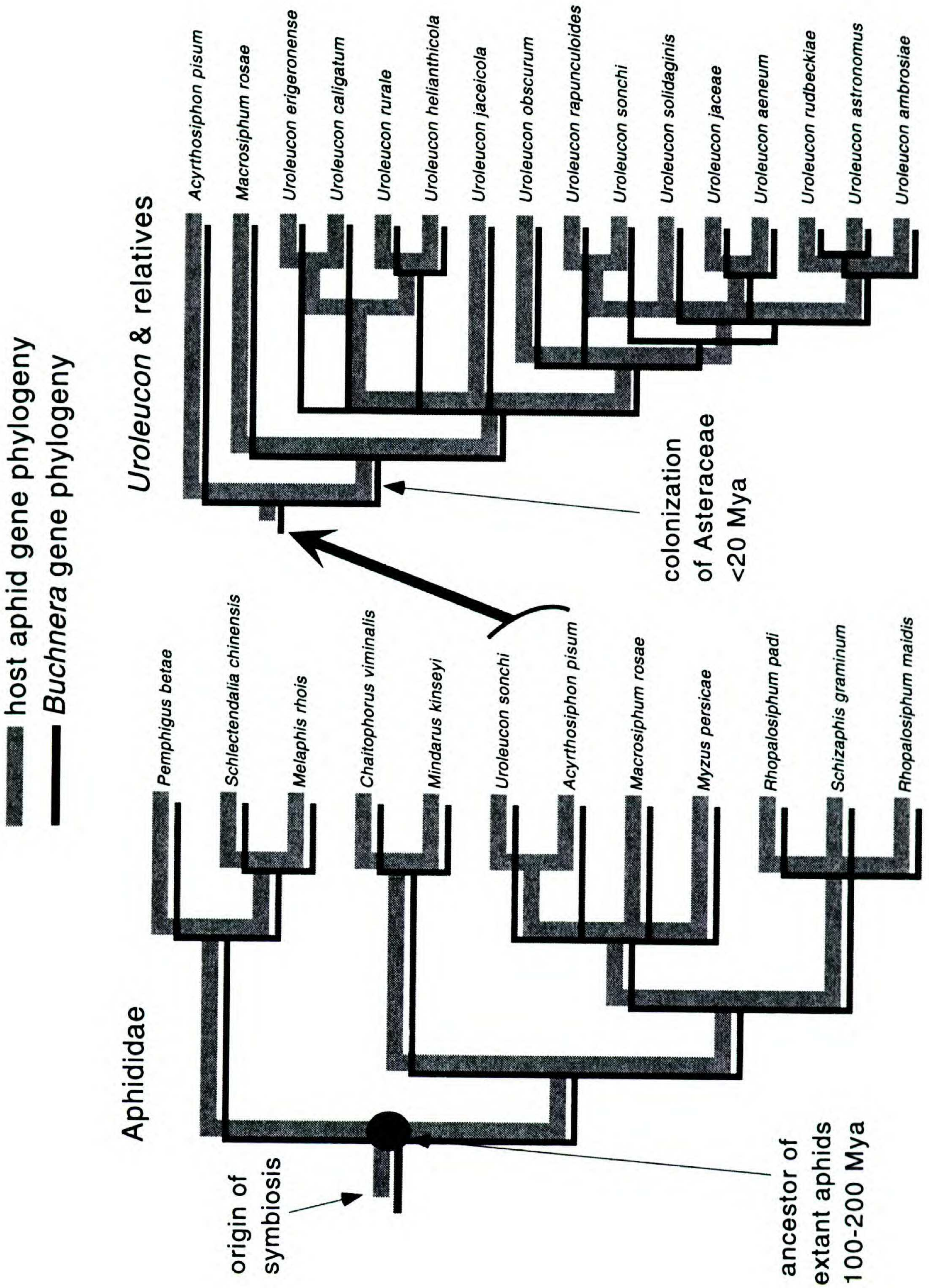
#### CODIVERSIFICATION

Phylogenetic analyses for *Buchnera* and aphids support strict cospeciation, with strictly vertical transmission over at least 100 million years (Fig. 3). This conclusion is based on analyses at several phylogenetic depths. First, phylogenies based on representatives from the superfamily Aphidoidea are completely concordant with phylogenies of their corresponding endosymbionts, based on deeply diverging lineages (Munson et al., 1991b; Moran et al., 1993). However, phylogenetic studies of such divergent groups might not reveal horizontal transfer if it has occurred between related aphid lineages, such as members of the same aphid tribe or genus. Phylogenetic concordance of *Buchnera* and 15 host species within the aphid genus *Uroleucon* was examined to determine if transfer among related host insects has taken place (Clark et al., 2000; Fig. 3). These aphids are restricted to plants within the Asteraceae, sometimes use the same host plant taxon, and often share natural enemies including parasitoid wasps that might act as vectors for symbiotic bacteria (Moran et al., 1998). Even these

ecologically and phylogenetically related hosts showed strict codivergence with no horizontal transmission of *Buchnera* (Clark et al., 2000). Finally, molecular phylogenetic studies within a single aphid species, *Uroleucon ambrosiae*, indicate strict codivergence of symbiont genes and mitochondrial genes, indicating that even intraspecific transfer of *Buchnera* is absent (Funk et al., 2000). Since even rare instances of transfer would result in major discrepancies between phylogenies of host and symbiont, these combined results suggest that *Buchnera* has completely lost the capacity to live outside hosts and/or to invade new hosts.

#### AGE OF ENDOSYMBIOSES IN STERNORRHYNCHA

The shared ancestor of the superfamily Aphidoidea lived 100–200 million years ago (Heie, 1987). The observation of congruent phylogenies for aphids and *Buchnera* implies that the original infection is at least this old. Since the large majority of modern Sternorrhyncha have endosymbionts and feed on plant phloem sap, it seems likely that the common ancestor of the group also possessed endosymbionts. However, the current distribution of symbiont lineages at a higher taxonomic level, i.e., among families of Sternorrhyncha, indicates that there have been numerous independent infections, possibly involving replacements of ancestral symbionts with novel ones (Fig. 2). For example, some of the symbionts characterized for the Coccoidea, the sister group to Aphidoidea, are in the beta-Proteobacteria (Munson et al., 1992) although other groups of bacteria appear to be present in at least



some coccoid species (Kantheti et al., 1996; Fukatsu & Nikoh, 2000). The Aleyrodoidea and the Psylloidea, sister clades within Sternorrhyncha, each contain a clade of gamma-Proteobacteria that are possibly sister clades to one another but not to *Buchnera* (Clark et al., 1993; Spaulding & von Dohlen, 1998).

Based on recent studies, phylogenetic relationships are congruent for the Psylloidea and their corresponding symbionts (Thao et al., 2000a). This result implies an ancient infection, since the Psylloidea are probably of similar age to the Aphidoidea. The infection may have predated divergence of Aleyrodoidea from Psylloidea; however, current information based on ribosomal sequences is not sufficient to fully resolve the sister group of psyllid symbionts.

#### DISTINCTIVE CHARACTERISTICS OF ENDOSYMBIONTS AND COADAPTATION WITH HOSTS

The 100 million years or more that *Buchnera* has resided in aphids implies more than a billion bacterial generations. How has it diverged from its nonsymbiotic ancestor? Extensive characterization of *Buchnera* from the aphid *Schizaphis graminum* (Rondani) combined with comparative studies of symbionts of some other aphid hosts indicate that *Buchnera* is closely related to *Escherichia coli* but shows a very divergent overall pattern of genome evolution (Baumann et al., 1999). The relationship to *E. coli* and other enteric bacteria has been verified repeatedly by sequence comparisons of homologous genes: almost all of the loci sequenced so far for *Buchnera* have clear homologs in the *E. coli* genome, in agreement with initial results based on 16S rDNA sequences (P. Baumann et al., 1999; Shigenobu et al., 2000).

One indicator of a distinctive pattern of genome evolution is evident from the extremely small genome size. The genome of *Buchnera* of *Acyrtosiphon pisum* is only about 640 kilobases and contains 590 genes (Charles & Ishikawa, 1999; Shigenobu et al., 2000), about a seventh of the size of the genome of *E. coli* at about 4500 kilobases and over 4000 genes. Similar sizes are found in *Buchnera* of other Aphidoidea (Wernegreen et al., 2000). An implication is that *Buchnera* underwent genome reduction, losing most of its DNA and most genes in the course of its evolution from a free-

living ancestor. This resembles the situation in phylogenetically diverse intracellular pathogenic bacteria, which have repeatedly evolved small genomes (Andersson & Kurland, 1998; Moran & Wernegreen, 2000). Because bacterial genomes are mostly composed of functional genes, an implication is that *Buchnera* has lost most ancestral loci and corresponding functional capabilities.

A second distinctive aspect of genome evolution is revealed in the presence of plasmids bearing genes for biosynthesis of amino acids required by the host. Plasmid location of these genes is not typical of free-living bacteria, and the plasmid location allows increased gene copy number and overproduction of the gene product. *Buchnera* of some hosts, including members of the large family Aphididae, possess two plasmid types that are involved in production of tryptophan and leucine respectively (Lai et al., 1994; Bracho et al., 1995). On one of these, *trpEG*, the rate-limiting genes for tryptophan biosynthesis, are amplified as tandem repeats. For example, in the host *Schizaphis graminum*, each plasmid has four repeats and about four plasmids are present for each chromosomal copy, resulting in an effective 16-fold amplification. On the other plasmid, the four genes underlying leucine biosynthesis are present as single copies, along with replication genes and an unknown conserved open reading frame (van Ham et al., 1997). The plasmid is present in multiple copies, with an effective 20-fold amplification in *Buchnera* of *S. graminum* relative to genes on the main bacterial chromosome. The two types of plasmids are entirely different in organization, implying that they originated independently. Within each type, gene rearrangements and sequence evolution have occurred, resulting in differences among host taxa (Rohbakhsh et al., 1996; van Ham et al., 1997; Silva et al., 1998; L. Baumann et al., 1999).

#### COEVOLUTION AND PLASMIDS IN APHID SYMBIONTS

Both the leucine plasmid and the tryptophan plasmid are interpreted as adaptive modifications of the *Buchnera* genetic system that enable improved growth and reproduction of hosts. Bacterial plasmids are usually thought to be involved in gene transfer among bacterial strains or species. However, in the case of the *Buchnera* plasmids, phylogenetic evidence, based on sequences of genes

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Figure 3. Phylogenetic congruence of aphids and their endosymbiotic bacteria, *Buchnera*, at the level of the family Aphididae and at the level of an ecologically homogeneous clade of aphid hosts, the genus *Uroleucon* and relatives. Trees are redrawn from Munson et al., 1991b; Moran et al., 1993; Moran et al., 1998; and Clark et al., 2000.

from both plasmid types, the bacterial chromosome, and the aphid mitochondrion, indicates strict vertical transmission of the plasmids within the clade corresponding to the aphid family Aphididae (Rohbakhsh et al., 1997; Silva et al., 1998; L. Baumann et al., 1999), within the genus *Uroleucon* (Wernegreen & Moran, 2001), and within the species *Uroleucon ambrosiae* (Funk et al., 2000). In other words, not only is *Buchnera* itself entirely vertically transmitted, its plasmids also are, resulting in complete linkage of the plasmid-borne genes with genes on the bacterial chromosome and genes on the aphid mitochondrial chromosome. This situation might facilitate coadaptation of genes in different locations and improved coordination of expression of biosynthetic genes important to the fitness of aphid hosts.

#### SELFISH SYMBIONTS

Because *Buchnera* is strictly vertically transmitted, the possibilities for the establishment of symbiont traits that harm host fitness are quite limited. A bacterial mutant that replicated faster but provisioned hosts less well might spread within the population of bacteria in a single host and might then preferentially infect progeny. However, hosts bearing symbionts with "selfish" traits, i.e., those that benefit bacterial replication at the expense of host fitness, would experience negative selection within host populations even if bacteria are favorably selected within individual hosts. However, in small host populations that experience genetic drift, there is some potential for the spread of selfish traits despite counter selection at the host level. This is especially true if effects on hosts are small and if such alleles originate frequently through mutation (Michod, 1997; Rispe & Moran, 2000). Frequency of selfish mutations will depend, in part, on the size of the mutational target. Most mutations knocking out functionality of genes that underlie biosynthesis of host nutrients might have selfish effects, since they will compromise host nutrition but could improve rate of replication of the individual mutant cell lineage. The proportion of the genome underlying amino acid biosynthesis is substantial: based on the combined length of genes for synthesis of essential amino acids and on the full genome size (Charles & Ishikawa, 1999) this proportion could be as much as 10% or more. A high rate of origin of selfish mutations, combined with a short-term selective advantage within hosts, might result in a constant threat to aphid fitness.

#### DEGENERATIVE SYMBIONTS?

Selection at the level of host individuals favors mutualistic, and functional, symbionts. However, such selection will be limited in effectiveness. First, since selection occurs mostly at the level of the animal host, the effective population size will be relatively small resulting in more genetic drift. This is because animal populations are relatively small (as compared to populations of most free-living bacteria). Furthermore, the symbionts forego any recombination with bacteria from other hosts, a conclusion strongly supported by phylogenetic results that support vertical transmission even for plasmid-borne genes are strictly vertically transmitted (Funk, Wernegreen & Moran, unpublished results).

A variety of evidence based on gene sequences suggests that *Buchnera* and other symbionts have been subject to the fixation of slightly deleterious mutations through genetic drift. First, rates of DNA sequence evolution are increased in symbionts, as expected if a larger proportion of mutations are fixed rather than eliminated through purifying selection (Moran, 1996; Spaulding & von Dohlen, 1998; Peek et al., 1998; Wernegreen & Moran, 1999). This acceleration is found at all loci analyzed and, in genes encoding polypeptides, is concentrated at sites causing amino acid substitutions, that is, sites likely to be subject to purifying selection (Moran, 1996; Wernegreen & Moran, 1999). The sequence changes lead to decreased stability in secondary structure of gene products (Lambert & Moran, 1998), as expected if they are slightly deleterious. These patterns appear to characterize many endosymbiont groups that are maternally transmitted in invertebrate hosts (Moran, 1996; Lambert & Moran, 1998; Peek et al., 1998). Another common feature of endosymbionts is the overproduction of the stress protein GroEL (Sato & Ishikawa, 1997; Aksoy, 1995). Bacterial GroEL functions to fold proteins that have lost their functional conformation (Ewalt et al., 1997), and it is typically overproduced in response to stress such as heat. Constitutively high levels in endosymbionts may function to maintain functionality of polypeptides that are less stable due to multiple amino acid substitutions resulting from mutation fixation through genetic drift (Moran, 1996). Finally, the small genome sizes of endosymbionts may be the consequence in part of genetic drift causing the fixation of mutations that eliminate function of genes that are beneficial but not essential. Once fixed, silenced alleles would disappear through neutral deletions.

VARIABLE HOST NUTRITION AND VARIATION IN  
SYMBIONT PROVISIONING CAPACITY

Oddly, some *Buchnera* have undergone secondary loss of the extra copies of genes for biosynthesis of essential amino acids. Apparently, the ability to produce large amounts of these nutrients has been eliminated. In the case of *trpEG*, the genes for tryptophan production, some of the tandem repeats are eliminated as pseudogenes (gene copies with strong homology to functioning copies but with multiple frameshifts and stop codons in the coding region). In the case of the genes for synthesis of leucine, the plasmid copy number is reduced. An example of this situation is in *Buchnera* of *Diuraphis noxia*, an aphid that causes extensive damage to its *Triticum* host (wheat) and related grasses. In *D. noxia*, there are only one or two copies of plasmid *trpEG* and of *leuABCD* relative to each chromosomal gene; in contrast, other Aphididae have 16 to 24 copies for both sets of plasmid-encoded biosynthetic genes (Thao et al., 1998). Although this situation is unusual, it is not unique; other instances of reduced amplification level for amino acid biosynthetic genes are known in *Buchnera* of other aphid species (e.g., Baumann et al., 1997).

Are these reductions in gene copy number adaptive, potentially reflecting decreased host need for symbiont provisioning? Or are they maladaptive at the level of the host organism, potentially resulting from genetic drift or even selection at the intrahost level? Unusual enrichment of ingested phloem sap might lead to reduced dependence on symbiont biosynthesis and even selection to lower production levels by symbionts. In fact, studies on the nutrient composition of phloem sap ingested by *Diuraphis noxia* indicate that this species does ingest a diet enriched in essential amino acids. This enrichment is an effect of aphid feeding, which causes a characteristic damage to the infested plant and an increase of essential amino acids in the ingested phloem sap (Telang et al., 1999). The observed loss of gene copies may relate to this nutritional enrichment (Wernegreen & Moran, 2000). Nonetheless, haemolymph levels of leucine and tryptophan are unusually low in *D. noxia*, suggesting that these nutrients may be limiting to growth despite increased concentrations in ingested sap (Wernegreen & Moran, 2000). Like *D. noxia*, *Schizaphis graminum* causes damage on grass host plants that results in increases in amino acid concentrations in the diet. *Buchnera* of *S. graminum* do not show a loss of amplified gene copies, and *S. graminum* haemolymph is not low in tryptophan and leucine. Furthermore, *S. graminum* shows a much higher

growth rate than does *D. noxia*. Thus, it appears that the decreased biosynthetic capability of *Buchnera* of *D. noxia* does correspond to lower tryptophan and leucine availability in *D. noxia*, and to a lower growth rate of the host, but whether this decrease is maladaptive for the host is not clear. Possibly, the low rate of reproduction and growth of *D. noxia* is part of a life history strategy that is connected with its habit of forming small sedentary colonies within curled leaves of hosts. Pseudogenes for *trpEG* are widespread among *D. noxia* populations and have arisen independently in other *Diuraphis* species that inflict similar damage on their host plants (Wernegreen & Moran, 2000).

SECONDARY SYMBIONTS

Many species of Sternorrhyncha have more than one maternally transmitted symbiont, within the same individual insect. In aphids, symbionts other than *Buchnera*, called secondary or accessory symbionts, live outside the bacteriocytes, often in the nearby sheath cells (Buchner, 1965). Whether they are mutualistic or possibly pathogenic is not known. Associations are maternally transmitted but are evolutionarily less long-lived, with sporadic distribution among related species or even within a species (e.g., Chen & Purcell, 1998; Sandström et al., 2001). Some horizontal transmission also occurs, based on the presence of extremely closely related symbionts in phylogenetically divergent hosts (Chen & Purcell, 1998), but the mechanism of transfer is not known. Secondary symbionts also occur in psyllids (Fukatsu & Nikoh, 1998; Spaulding & von Dohlen, 1998) and in whiteflies (Clark et al., 1993). Secondary symbionts sometimes do not show the unusually fast sequence evolution and biased base composition that is typical of primary symbionts; their sequences may resemble those of characterized free-living bacteria such as the enterics (Chen & Purcell, 1998; Clark et al., 1993). However some secondary symbionts, perhaps those with longer histories as symbionts, do show some tendency toward accelerated evolution and biased base composition (Spaulding & von Dohlen, 1998; Fukatsu & Nikoh, 1998; Thao et al., 2000b).

SPECULATIONS ON THE EVOLUTION OF  
ENDOSYMBIOSES

Endosymbioses may initially become established on the basis of nutritional benefits to hosts, explaining their concentration in animal clades, such as Sternorrhyncha, that live on restricted diets (Buchner, 1965). If infections benefit host fecundity, then infected maternal lineages will spread; similarly,

symbiont lineages with traits benefitting host fecundity will spread at the expense of symbiont lineages that lack these benefits. The latter is illustrated in *Buchnera* by the amplification of amino acid biosynthetic genes on plasmids: this feature apparently evolved few times and must have become fixed through selection at the level of host individuals. Subsequently, as symbionts become more integrated into the development and physiology of hosts, hosts may evolve dependencies on their bacteria that do not involve nutrition. This possibility, that hosts evolve increasing dependence on their long-term symbionts, is supported by repeated observations that aphids deprived of *Buchnera* cannot reproduce, even when limiting nutrients are supplied in artificial diets (Douglas, 1998). Although the association is beneficial and even obligate for hosts, the symbionts themselves may become less functional due to a large effect of genetic drift on their evolution, with the consequent fixation of mildly deleterious mutations.

The genome size of *Buchnera* is near the lower limit observed in bacteria (Maniloff, 1996; Charles & Ishikawa, 1999; Wernegreen et al., 2000). Most small genome bacteria studied so far are pathogens that form chronic associations with animal hosts. These pathogenic bacteria have lost many biosynthetic pathways, including almost all amino acid biosynthetic pathways, obtaining needed nutrients from host cells. In contrast, genetic characterization of *Buchnera* indicates that multiple amino acid biosynthetic pathways are retained (P. Baumann et al., 1999; Shigenobu et al., 2000). An implication is that *Buchnera* evolved from a free-living bacterium that became endosymbiotic and mutualistic as it lost genes: it did not evolve from an intracellular bacterium already possessing a reduced genome. The amino acid biosynthetic genes are too numerous and too dispersed in the genome to have been re-acquired after being lost in a pathogenic ancestor. Thus, the idea that mutualistic bacteria arise from long-term pathogens can be discounted in this case and perhaps in others (Moran & Wernegreen, 2000).

Do other, independently acquired, symbionts of phloem-feeders show the same patterns discovered for *Buchnera*? The answer to this question will become evident during the next few years, through genome characterization of other symbionts. Initial results, for symbionts of psyllids, indicate some striking similarities (Spaulding & von Dohlen, 1998; Thao et al., 2000a).

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